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The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties

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We demonstrate here that the human oestrogen receptor (hER) cDNA clone pOR8 obtained from MCF-7 cells contains an artefactual point mutation which results in the substitution of a valine for a glycine at amino acid position 400 (Gly-400 → Val-400). This mutation in the hormone binding domain of the cloned hER destabilizes its structure and decreases its apparent affinity for oestradiol at 25°C, but not at 4°C, when compared with the wild-type hER with a Gly-400.

Key words: cDNA/reverse transcriptase error/oestrogen receptor mutant/breast cancer cells/hormone-binding domain

Introduction

The oestrogen receptor (ER) belongs to the nuclear receptor family, a class of DNA binding proteins that act as ligand-activated enhancer factors and includes the steroid/thyroid hormone receptors, vitamin D3 and retinoic acid receptors (see Green *et al.*, 1986; Petkovich *et al.*, 1987; Evans, 1988; Green and Chambon, 1988; Gronemeyer *et al.*, 1988; and refs therein). We isolated the human ER (hER) cDNA clone pOR8 from a cDNA library of the human breast cancer cell line MCF-7 (Walter *et al.*, 1985; Green *et al.*, 1986). This cDNA, which was sequenced both by us (Green *et al.*, 1986) and by others (Greene *et al.*, 1986), encodes the entire open reading frame of hER mRNA. Its transient expression in transfected HeLa cells resulted in the synthesis of a protein which bound oestradiol at 4°C with the same affinity ($K_D \simeq 0.4$ nM) as the endogenous MCF-7 cell hER (Green *et al.*, 1986). The chicken ER (cER) cDNA was also cloned in our laboratory from laying-hen oviduct RNA (Krust *et al.*, 1986). Comparison of cER and hER sequences revealed six regions of homology, denoted A–F (Krust *et al.*, 1986). Functional analyses of hER cDNA mutants demonstrated that regions C and E, which are highly conserved between human and chicken, correspond to the DNA and hormone binding domains respectively (Kumar *et al.*, 1986; Green and Chambon, 1987; Kumar *et al.*, 1987; Kumar and Chambon, 1988; Green *et al.*, 1988a; Mader *et al.*, 1989; Webster *et al.*, 1989). The A/B region, which is less conserved, is important for efficient stimulation of transcription from certain oestrogen-responsive promoters (Kumar *et al.*, 1987; Tora *et al.*, 1988).

In the course of a study aimed at comparing the transcriptional activation function of recombinant hER and cER, we found that they may differ in their affinity for oestradiol (our unpublished results). We have investigated the basis for this difference, and report here that there is most probably an artefactual point mutation in the hER cDNA clone pOR8 which results in the presence of a valine instead of a glycine at position 400. hER with a Val-400 exhibits an apparent decreased affinity for oestradiol at 25°C (but not at 4°C), when compared with the wild-type recombinant hER with a Gly-400. The latter, however, binds the hormone at 25°C with the same affinity as cER or MCF-7 cell hER.

Results

Apparent oestrogen-independent stimulation of transcription by oestrogen receptors with Gly-400 instead of Val-400

We first compared the transcriptional activation characteristics of hER (Green *et al.*, 1986; Kumar *et al.*, 1987) with that of cER (Krust *et al.*, 1986) using HeLa cells transiently co-transfected with the oestrogen-responsive reporter plasmid vit-tk-CAT and the appropriate vectors expressing either hER (HEO, which contains the cDNA clone pOR8, see Walter *et al.*, 1985; Green *et al.*, 1986; Kumar *et al.*, 1987) or cER (CEO, Materials and methods). To our surprise CEO activated transcription to some extent (~15% of the activation seen in the presence of 10^{-8} M oestradiol) without adding oestradiol (E2) to the phenol red-free and hormone-stripped culture medium (compare lanes 13 and 14 in Figure 1), whereas no activation was seen with HEO in the absence of E2 in agreement with our previous reports

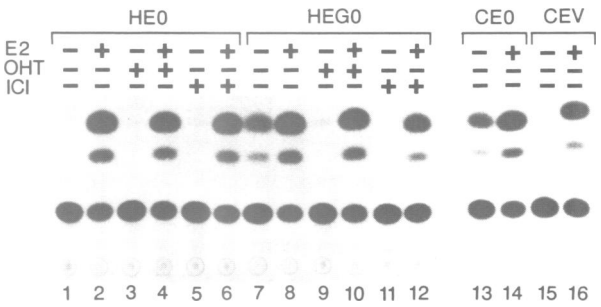

Fig. 1. Effect of the glycine 400 to valine mutation in human and chicken ER on activation of transcription. HeLa cells were transfected with 50 ng of human ER or chicken ER expression vectors as indicated, together with 1 μg of vit-tk-CAT reporter plasmid, and the internal control β-galactosidase expression plasmid pCH110 (3 μg) and carrier DNA (Bluescribe M13+) up to 20 μg total DNA. Cultures were maintained for 48 h in the absence (−) or in the presence (+) of 10^{-8} M oestradiol (E2), and then assayed for CAT enzyme activity after normalization for β-galactosidase activity (Webster *et al.*, 1988). Anti-hormones were added as indicated using the following concentrations: 4-hydroxy-tamoxifen (OHT), 10^{-8} M; ICI 164,384 (ICI), 10^{-7} M.

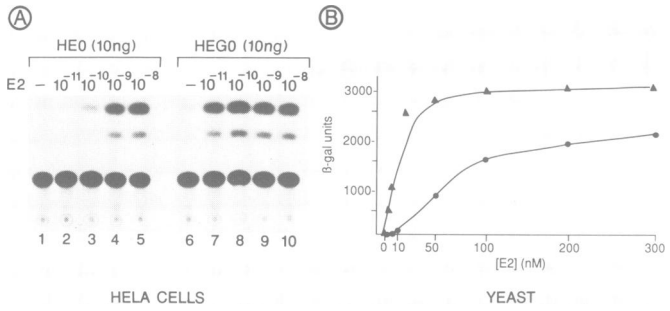

Fig. 2. Effect of increasing oestradiol concentrations on activation of transcription by the wild-type (Gly-400) and mutant (Val-400) hERs. (**A**) HeLa cells were transfected with 10 ng of the corresponding hER expression vectors, HEO (lanes 1–5) or HEGO (lanes 6–10), together with 1 μg of vit-tk-CAT reporter plasmid, 3 μg of the β-galactosidase control plasmid pCH110 and Bluescribe M13+ DNA up to 20 μg total DNA. The cells were maintained for 48 h in the absence (−) or in the presence (+) of different oestradiol (E2) concentrations as indicated and assayed for CAT enzyme activity. (**B**) Yeast cells were transformed with pYERE1/HER (closed circles), expressing the mutant Val-400 hER, and with pYERE1/HEGO (closed triangles) expressing the wild-type Gly-400 hER. These vectors also express the *Escherichia coli lacZ* gene under the control of a promoter that contains an ERE located upstream of the *GAL1* gene TATA-box (Metzger *et al.*, 1988). Yeast cells were grown as described (Metzger *et al.*, 1988) and at OD_{600} = 0.6 oestradiol (E2) was added as indicated. Two hours later, the yeast cells were harvested and the β-galactosidase assay was carried out.

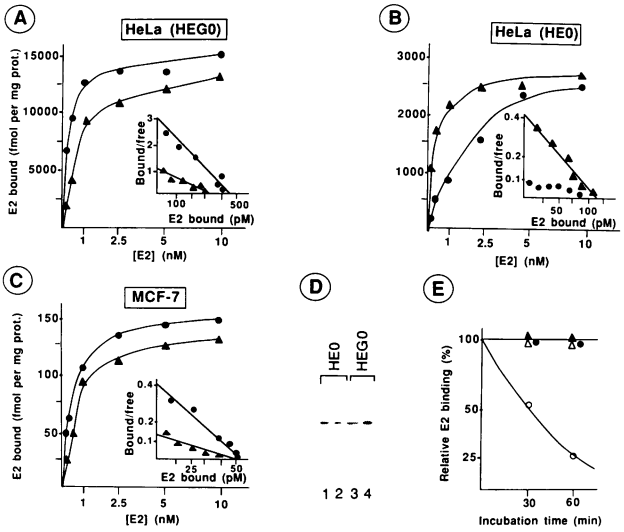

Fig. 3. The apparent affinity for oestradiol of hER encoded by HEO is altered at 25°C. Cytosolic extracts prepared from HeLa cells transfected with either 10 μg HEGO (**A**) or 10 μg HEO (**B**), or from MCF-7 cells (**C**) were either incubated for 2 h at 25°C (closed circles) or overnight at 0°C (closed triangles) in the presence of [^{3}H]17β-oestradiol (E2) with or without a 200-fold excess of unlabelled E2. Unbound steroid was removed using dextran-coated charcoal. Bound steroid was measured by scintillation counting and specific binding was calculated after subtraction of non-specific binding (Kumar *et al.*, 1986). The inserts show the Scatchard plots of these data. (**D**) Western blot analysis of cytosolic extracts before (**lane 1**, HEO; **lane 3**, HEGO) and after (**lane 2**, HEO; **lane 4**, HEGO) incubation at 25°C for 2 h, using the monoclonal antibodies H222 and D75 (Greene *et al.*, 1984). (**E**) Cytosolic extracts prepared from HeLa cells transfected with 10 μg HEGO or HEO, were either incubated at 25°C without oestrogen (open triangles for HEGO, open circles for HEO), or in the presence of 10 nM [^{3}H]17β-oestradiol (closed triangles for HEGO, closed circles for HEO). After a 30- or 60-min incubation period, 10 nM [^{3}H]17β-oestradiol was added to the minus hormone samples and all samples were further incubated for 2 h at 25°C. Non-specific binding was determined in parallel incubations in the presence of a 200-fold excess of unlabelled E2. Receptor-bound steroid was determined as above. One hundred per cent oestrogen binding corresponds to extracts incubated with labelled oestradiol for 2 h.

(see, for example, Kumar *et al.*, 1987) (compare lanes 1 and 2 in Figure 1).

To localize the cER region responsible for this transcriptional activation in the apparent absence of E2, we constructed a series of hER–cER chimeras. A test of their transcriptional activation function, using the same transient transfection system as above, revealed that the conserved segment of the hormone-binding domain of cER from amino acids 372 to 546 was responsible for the difference between HEO and CEO (data not shown). Interestingly, a comparison between the sequence of the hER cDNA clone pOR8 (which has been used in the construction of HEO) and the sequence of the exons of the hER gene cloned from placental DNA has recently revealed a single base difference within the codon for amino acid 400 (Ponglikitmongkol *et al.*, 1988). A valine is encoded in the pOR8 cDNA clone, whereas a glycine is encoded at the corresponding position in the human genomic sequence [Val-400 (GTG) → Gly-400 (GGG)]. Moreover a glycine residue is present at the equivalent position in the chicken (Gly-394; Krust *et al.*, 1986), mouse (Gly-404; White *et al.*, 1987), rat (Gly-405; Koike *et al.*, 1987) and *Xenopus* (Gly-392; Weiler *et al.*, 1987) ER sequences, suggesting that the hER cDNA clone pOR8 may not be 'wild-type' at this position.

To test whether the presence of Val-400 instead of Gly-400 could be responsible for the different oestradiol requirement of HEO and CEO for activation of transcription, site-directed mutagenesis was used to replace Val-400 of HEO with a glycine residue (HEGO expression vector) and, reciprocally, to replace the corresponding Gly-394 of CEO with a valine residue (CEV expression vector). In support of the above hypothesis, HEGO activated transcription to approximately the same extent as CEO in the absence of oestrogen (compare lanes 1, 7 and 13 in Figure 1), whereas CEV lost the CEO ability to activate transcription in the absence of oestradiol and behaved like HEO (compare lanes 1, 13 and 15).

Oestrogen antagonists were used to investigate whether the transcriptional activation observed with HEGO and CEO in the absence of added oestradiol could be due to residual levels of oestrogens remaining in the 'stripped' culture medium from which steroid hormones should have been removed by treatment with dextran-coated charcoal. Both 10^{-8} M 4-hydroxy-tamoxifen (OHT) (Figure 1, compare lanes 7 and 9) and 10^{-7} M ICI 164,384 (ICI) (compare lanes 7 and 11) suppressed the apparently oestrogen-independent activity of HEGO. Note that in the presence of oestradiol at 10^{-8} M, similar additions of OHT at 10^{-8} M and ICI 164,384 at 10^{-7} M had very little effect on oestradiol induction (lanes 10 and 12 respectively).

The presence of Val-400 instead of Gly-400 apparently decreases the affinity of hER for oestradiol at 25°C, but not at 4°C

The above results suggested to us that ERs with Gly-400 (HEGO, CEO) may exhibit a higher affinity for oestrogens than ERs with Val-400 (HEO, CEV), and therefore could be activated to some extent by residual oestrogens present in the 'stripped' culture medium. Both *in vivo* transcriptional activation experiments and *in vitro* hormone-binding studies were performed to investigate this possibility. *In vivo*

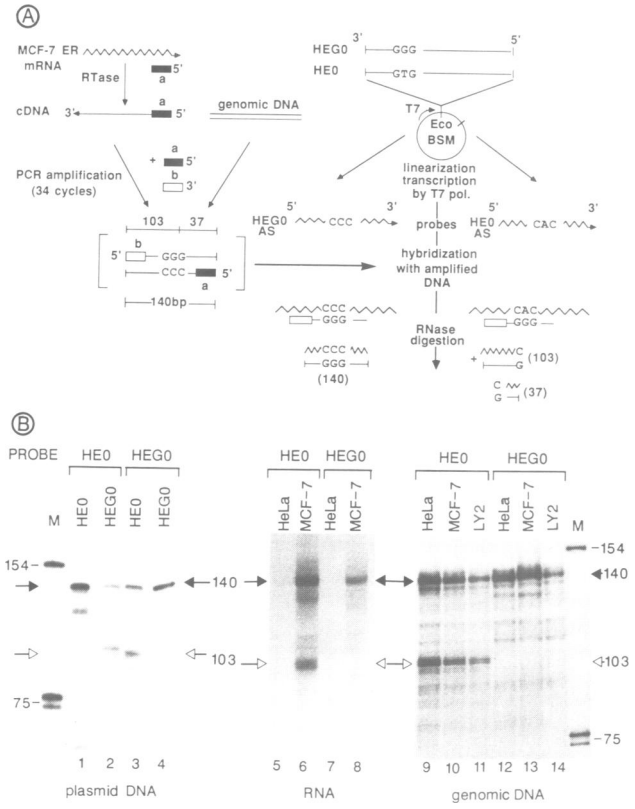

Fig. 4. (**A**) Schematic representation of PCR amplification and RNase mapping analysis to detect single-base substitutions in hER mRNA and genomic DNA samples from ER positive and negative cells. Single-stranded cDNA was synthesized using total cell RNA, reverse transcriptase (RTase) and oligonucleotide 'a' (Materials and methods). Sequences corresponding to the 140 bp-long exon 5 of the hER were PCR amplified from cDNA and genomic DNA, using oligonucleotides 'a' and 'b' as primers (Materials and methods). Antisense (AS) HEGO and HEO probes were synthesized using the T7 polymerase (pol) and linearized Bluescribe M13+ (BSM) plasmids containing the hER cDNA sequences. AS probes were hybridized to amplified DNA samples, digested with RNase and the samples were analysed on denaturating PAGE. Protection by the whole exon 5 of hER resulted in a 140 nucleotide-long fragment, whereas the presence of a mismatch at amino acid residue 400 results in two fragments (103 and 37 nucleotides long). In the latter case the RNase digestion was never complete, resulting in the presence of a 140 nucleotide-long band even in the positive controls. Positive controls were carried out using HEGO and HEO plasmid DNA samples (not shown in the scheme, but see panel B, lanes 1–4). (**B**) HEO (**lanes 1** and **3**) and HEGO (**lanes 2** and **4**) plasmid samples amplified by PCR were hybridized with either the HEO AS probes (**lanes 1** and **2**) or the HEGO AS probe (**lanes 3** and **4**) and digested with RNase. HeLa cell (as negative control, **lanes 5** and **7**) and MCF-7 cell (**lanes 6** and **8**) PCR-amplified cDNA samples were hybridized with either the HEO AS probe (**lanes 5** and **6**) or HEGO AS probe (**lanes 7** and **8**) and digested with RNase. HeLa cell (**lanes 9** and **12**), MCF-7 cell (**lanes 10** and **13**) and LY2 cell (**lanes 11** and **14**) PCR-amplified genomic DNA samples were hybridized with either the HEO AS probe (**lanes 9–11**) or the HEGO AS probe (**lanes 12–14**) and digested with RNase. The filled arrowheads indicate the 140 nucleotide-long full-length protected fragment, whereas the open arrowheads point to the 103 nucleotide-long fragment that is formed when RNase cuts the mismatch in the hybrids. M, mol. wt marker.

transcriptional activation was analysed both in HeLa cells using the vit-tk-CAT reporter gene and only 10 ng of ER expression vector (Figure 2A), and in yeast using the β-galactosidase assay system described by Metzger *et al.* (1988) (Figure 2B). In both cases maximal activation of transcription was achieved at a lower E2 concentration with HEGO than with HEO. In HeLa cells, maximal CAT activity was observed at 10^{-10} M E2 with HEGO, and at 10^{-9} – 10^{-8} M E2 with HEO (Figure 2A, compare lanes 1–5 with lanes 6–10). For *in vivo* transcriptional activation studies in yeast, the Val-400-containing hER sequence present in the pYERE1/HER plasmid (Metzger *et al.*, 1988) was replaced with the corresponding Gly-400 hER sequence to yield the plasmid pYERE1/HEG. Half-maximal β-galactosidase activity was achieved at ~5 nM E2 with the Gly-400 construct pYERE1/HEG and at ~50 nM E2 with the Val-400-containing construct at pYERE1/HER (Figure 2B and data not shown). It is particularly noteworthy that, in both cases, no β-galactosidase activity could be detected in yeast in the absence of oestradiol addition, supporting the above suggestion that the transcriptional activation observed in HeLa cells with HEGO in the absence of oestradiol was due to low levels of oestrogens remaining in the 'stripped' culture medium.

The affinity for oestradiol of hERs containing either Val-400 (HEO) or Gly-400 (HEGO) was then determined *in vitro* at both 4°C and 25°C, and compared with that of the endogenous MCF-7 cell hER (Figure 3A–C). Cytosol extracts were prepared from HeLa cells transfected with either HEO or HEGO and from the breast cancer cells MCF-7. Both HEGO and HEO hERs expressed in HeLa cells (Figure 3A,B) exhibited at 4°C the same oestradiol affinity as MCF-7 hER (K_D = 0.35 ± 0.05 nM, Figure 3C), in agreement with our published study comparing HEO and MCF-7 cell hER (Green *et al.*, 1986). However, a striking difference in the apparent oestradiol affinity of HEGO and HEO was observed at 25°C. Whereas the affinities of HEGO and MCF-7 hER for oestradiol were similar (K_D = 0.14 ± 0.02 nM), the Scatchard representation of oestradiol binding by HEO at 25°C was not a straight line, precluding the determination of a K_D value. Interestingly, at saturating oestrogen concentrations (5–10 nM) HEO bound the same amount of E2 at 25°C as at 4°C (Figure 3B), suggesting that the presence of the hormone may stabilize HEO. Furthermore, at saturating oestradiol concentrations and irrespective of the incubation temperature, the HEGO cytosolic extract bound ~5-fold more oestradiol than the corresponding HEO extract (Figure 3A,B), even though both extracts contained approximately the same amount of hER protein as judged from Western blot analysis of these extracts carried out either before or after the 25°C incubation period (Figure 3D).

To investigate further whether the presence of oestradiol may stabilize the HEO protein at 25°C, we compared the stability of HEO and HEGO proteins at 25°C in the presence and absence of oestradiol. Cytosolic extracts of HeLa cells transfected with either HEO or HEGO were first incubated at 25°C without or with 10 nM labelled oestradiol (plus or minus an excess of cold E2) for 30 or 60 min. Labelled oestradiol (10 nM) plus or minus an excess of cold E2 was then added to the minus hormone samples, all the samples were further incubated for 2 h at 25°C, and specific E2 binding was determined (Figure 3E). HEGO bound oestradiol to the same extent, irrespective of whether it was preincubated with (closed triangles) or without (open triangles) E2, whereas HEO bound decreasing amounts of E2 when it was preincubated for 30 or 60 min without hormone (open circles). No such decrease in E2 binding was observed when E2 was added at time 0 (closed circles). These results indicate that HEO hER, but not the HEGO

hER, irreversibly lost its oestrogen-binding capacity during an incubation at 25°C. Note that this loss was not due to proteolysis (see above, Figure 3D).

Does the Val-400 encoded in the pOR8 hER cDNA result from a cloning artefact?

The above results suggested to us that the amino acid sequence of hER present in MCF-7 cells was identical to that predicted from the hER gene exonic sequences cloned from placental DNA. Thus, the Val-400 encoded in the cDNA clone pOR8 could be due to a point mutation that occurred during the cloning process. RNase mapping was used to investigate whether the mutation which results in a valine instead of a glycine (GGG→GTG) at position 400 could be detected in hER mRNA from MCF-7 cells. Sequences corresponding to the 140 bp-long exon 5 (Ponglikitmongkol *et al.*, 1988) were amplified from both cDNA synthesized from total MCF-7 cell RNA and genomic DNA, using the polymerase chain reaction (PCR) (Figure 4A). The amplified DNA fragments were then analysed by hybridization to antisense RNA probes (AS) using either the corresponding HEO or HEGO sequences as templates (Figure 4A, HEO AS and HEGO AS). The resulting hybrids were digested with RNase A and electrophoresed through denaturing polyacrylamide gels (Figure 4B). As expected, RNase cut the mismatch formed between the HEGO probe and DNA amplified from HEO plasmid DNA, and vice versa, to yield two 103 and 37 base-long fragments in addition to the undigested 140 base-long fragment (Figure 4A,B, lanes 1−4, and data not shown). On the other hand, the HEGO probe, hybridized to DNA amplified from cDNA derived from MCF-7 RNA (Figure 4B, lane 8) or from genomic DNA of either HeLa, MCF-7 or LY2 (another breast cancer cell line) cells (Figure 4B, lanes 12−14), yielded only one major 140 base-long protected fragment with no evidence of the 103 or 37 base-long fragments, indicating the presence of a codon for glycine in all cases (see Figure 4A). Even prolonged digestions at higher temperatures did not result in the appearance of the 103 and 37 base-long fragments, which would signify the presence of a valine codon (data not shown). However, hybridization of the HEO probe with the same samples yielded the two protected 103 and 37 base-long fragment nucleotides (Figure 4B, lanes 6 and 9−11, and data not shown). RNA from the ER-negative HeLa cells (Green *et al.*, 1986) did not result in any protected fragments (Figure 4B, lanes 5 and 7). These results indicate that the GTG sequence coding for a Val-400 in the cDNA clone pOR8 (and therefore in HEO) cannot be detected in bulk RNA from the breast cancer MCF-7 and LY2 cell lines, nor in human genomic DNA irrespective of its origin. Furthermore, we have sequenced the same region in three previously isolated MCF-7 hER cDNA clones [pOR3, pOR12 and pOR15 (Green *et al.*, 1987, and unpublished results)] and found, in agreement with the RNase mapping results, that all three contained the GGG sequence coding for a glycine at position 400 (data not shown).

Discussion

We demonstrate here that the human ER cDNA clone pOR8, which was obtained from MCF-7 cell RNA (Walter *et al.*, 1985; Green *et al.*, 1986), contains a point mutation at the position corresponding to the amino acid residue 400, resulting in the presence of a valine residue instead of the glycine residue that is encoded at this position in the hER gene and in the ER cDNA of different species (Krust *et al.*, 1986; Koike *et al.*, 1987; Weiler *et al.*, 1987; White *et al.*, 1987). That this mutation could be a cloning artefact is suggested by the observation that the valine codon GTG was not found in the sequence of three independent hER cDNA clones derived from MCF-7 cell RNA. The *in vitro* hormone-binding results also support the conclusion that the valine-containing hER (encoded by HEO) is different from the bulk of the MCF-7 cell endogenous hER. Although the oestradiol K_D values measured at 4°C were identical for HEO, HEGO (the glycine-containing hER) and the MCF-7 cell endogenous hER, the K_D for HEO determined at 25°C was different from that of HEGO and MCF-7 cell hER. Thus the presence of the Val-400 appears to correspond to a cloning artefact that occurred during the synthesis of the 2100 base-long pOR8 cDNA and may be due to a reverse transcriptase error (Roberts *et al.*, 1988 and refs therein), though we cannot exclude that the genome of a minor population of MCF-7 cells present in this mutation, nor that a minor fraction of hER mRNA could be mutated post-transcriptionally.

The mutation creating a valine in HEO affects the oestradiol-binding capacity of the receptor at 25°C, but not at 0°C. This is not due to a degradation of the protein during the oestradiol-binding incubation period (Figure 3D), but probably reflects an instability of the Val-400 hER at 25°C in the absence of oestradiol (Figure 3E), resulting in a loss of its capacity to bind oestradiol. *In vitro* incubation of HEO at 25°C without oestradiol reduced its hormone-binding capacity; however, when oestradiol was added at high concentrations, no loss of oestrogen binding was observed (Figure 3E). This instability of HEO hER at 25°C most likely accounts for the observation that ~5 times less oestradiol could be bound at optimal ligand concentration with extracts of cells transfected with HEO when compared to HEGO, even though the amounts of HEO and HEGO proteins were identical (Figure 3). This instability is probably also responsible for the reduced capacity of the HEO-encoded hER synthesized in yeast to bind oestradiol (Metzger *et al.*, 1988). According to secondary structure predictions (Gibrat *et al.*, 1987), the hER amino acid residue 400 may be situated in a β-turn between an α-helix and a hydrophobic β-strand. A search for this motif in a structural database and subsequent model building of the hER amino acid sequence around position 400 suggested that Pro-399 and Gly-400 occupy the two central positions of a type II β-turn, as often found in protein structures (Richardson, 1981). Thus the presence of a valine residue at position 400 could destabilize that part of the ER structure which may result in thermal denaturation at 25°C and thus loss of hormone binding. Whether the hER α-helix−β-turn−β-strand that contains the Gly-400 residue could be directly involved in oestradiol binding is unknown. That binding of the hormone stabilizes the hER Val-400 mutant suggests, however, that the three-dimensional structure of this hER region is influenced by the binding of the ligand.

The present results offer a satisfactory explanation for the observation that HEGO (wild-type hER) as well as the chicken oestrogen receptor, but not the mutant hER HEO, are able to activate transcription from the reporter gene via

vit-tk-CAT when the transfected HeLa cells are maintained in the steroid hormone-stripped culture medium (Figure 1). Due to its instability in the absence of oestradiol, HEO cannot bind effectively the oestrogens that are present in very limiting concentrations in the stripped culture medium, in contrast to HEGO, which is stable in the absence of oestradiol and therefore can activate transcription even under limiting oestrogen conditions. That residual oestrogens are in fact present in the stripped culture medium is indicated both by the inhibitory effect of the antagonists hydroxy-tamoxifen and ICI 164,348 on the apparently constitutive activity of HEGO in HeLa cells (Figure 1), and by the complete inactivity of the same receptor in yeast cells that are grown in a medium totally devoid of oestrogens (Figure 2B).

Finally, it is important to point out that a comparison of the DNA binding and transcriptional activation properties of the wild-type Gly-400 hER (HEGO expression vector) with those previously reported by our laboratory for the Val-400 hER [HEO expression vector (Green and Chambon, 1987; Kumar *et al.*, 1987; Kumar and Chambon, 1988; Tora *et al.*, 1988; Webster *et al.*, 1988, 1989; Mader *et al.*, 1989)] has not revealed, up to now, any significant differences (unpublished results from our laboratory).

Materials and methods

ER expression vectors and reporter plasmids

hER (Green *et al.*, 1986) and cER (Krust *et al.*, 1986) cDNA amino acid-coding sequences were subcloned into the *Eco*RI site of the eukaryotic expression vector pSG1 (Green *et al.*, 1988b) to yield HEO and CEO respectively. Single-stranded DNA was prepared and site-directed mutagenesis was performed as described (Grundström *et al.*, 1985) to generate the hER expression vector HEGO from HEO, and the cER expression vector CEV from CEO using the synthetic oligonucleotides:

5′-GGAGCACCCAGGGAAGCTACTGT-3′ and

5′-GGAACACCCAGTGAAGCTTTTATTT-3′

(the nucleotide changes are underlined) respectively. DNA sequences were verified by dideoxy-sequencing. Vit-tk-CAT (Klein-Hitpass *et al.*, 1986) and pYERE1/HER (Metzger *et al.*, 1988) have been described. pYERE1/HEG was constructed by inserting the hER cDNA sequences of HEGO into the parental vector described by Metzger *et al.* (1988).

Cell culture

HeLa and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with foetal calf serum (FCS). Five days before being used for hormone-dependent assay, they were transferred to phenol red-free DMEM medium supplemented with dextran-coated charcoal-treated FCS. LY2 is an MCF-7 cell variant, selected stepwise for stable resistance to the anti-oestrogen LY117018; it retains receptors for oestrogens (Bronzert *et al.*, 1985). LY2 cells were maintained on DMEM medium supplemented with dextran-coated charcoal-treated FCS and 1 μM tamoxifen. Yeast cells (TGY14.1 strain) were grown and transformed as described (Ito *et al.*, 1983; Metzger *et al.*, 1988).

Hormone-binding assay

HeLa cells (9 cm plates) were transfected with 10 μg receptor expression vectors and 10 μg carrier DNA (Bluescribe M13+, Stratagene) using the calcium phosphate precipitation technique (Banerji *et al.*, 1981). Cytosol extracts were prepared from transfected HeLa or MCF-7 cells and the hormone-binding assay was carried out as described (Green *et al.*, 1986), except that the homogenization buffer contained also protease inhibitors (2.5 μg/ml each aprotinin, leupeptin, chymostain and antipaïn), and that extract aliquots (50 μl) were incubated at 4°C overnight or at 25°C for 2 h with different concentrations of [^{3}H]17β-oestradiol (148 Ci/mmol) with or without a 200-fold excess of unlabelled 17β-oestradiol.

PCR amplification

Genomic DNA and RNA was isolated as described by Miller *et al.* (1988) and Groudine *et al.* (1981) respectively. RNA (5 μg) was used to prepare single-stranded cDNA as described by Huynh *et al.* (1985). The synthesis was primed with the synthetic 21mer oligonucleotide 'a' (see below). Sequences corresponding to exon 5 of the hER (Ponglikitmongkol *et al.*, 1988) were amplified using PCR, from either the single-stranded cDNA or from 1 μg genomic DNA in a 100 μl reaction volume containing: 10 mM Tris−HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM each dNTP, 1 μM each primer, 2.5 units *Taq* polymerase (Perkin-Elmer/Cetus). The primers were: 'a' 5′-CTGTCCAAGAGC-AAGTTAGGA-3′ and 'b' 5′-GGCTTTGTGGATTTGACCCTC-3′ (see also Figure 4A). The PCR (Saiki *et al.*, 1988) was performed using a Perkin-Elmer/Cetus Thermal Cycler and comprised an initial denaturation step for 90 s at 94°C, followed by 34 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 37°C and elongation for 3 min at 72°C. Each elongation period was successively increased by 4 s and the duration of the last elongation period was increased to 9 min.

RNase mapping

hER cDNA present in either HEO or HEGO was cloned into the *Eco*RI site of Bluescribe M13+ (Stratagene). Plasmids linearized with *Xmn*I were used in the T7 RNA polymerase *in vitro* transcription system according to instructions of the manufacturer to generate uniformly ^{32}P-labelled RNA probes. One microlitre of the PCR product was mixed with $\sim 5 \times 10^5$ c.p.m. of ^{32}P-labelled RNA probe in 15 μl of hybridization solution (400 mM NaCl, 10 mM Pipes, pH 6.5). The solution was heated to 85°C for 5 min and immediately transferred to a water bath maintained at 68°C and incubated for 12−16 h. RNase digestion and analysis of the protected fragments was performed as described by Winter *et al.* (1985).

Other methods

HeLa cell transfections using the calcium phosphate technique, CAT assays (Webster *et al.*, 1988), Western blot analysis (Green *et al.*, 1988a) and β-galactosidase assay (Metzger *et al.*, 1988) were performed as described.

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